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MUTAGENICITY TESTS OF FOREIGN SUBSTANCES

U. H. Ehling

Environmental Protection Agency
Research Triangle Park, North Carolina

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(Mutagenitätsprüfung von Fremdstoffen)

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U.H. Ehling

MUTAGENICITY TESTS OF FOREIGN SUBSTANCES[1]

In a population there is a balance between spontaneous mutability and selection. If this balance is disturbed by technical development, grave consequences result in the affected population due to the increase of hereditary diseases. /1/ Two factors can disturb the balance through an increase in the spontaneous mutation rate: radiation and harmful chemical substances.

Radiation-induced mutations in Drosophila melanogaster, the fruit fly, were discovered in 1927 by H.J. Muller. /2/ In exemplary fashion, the consequences of radiation-induced mutations in the population were examined by the International Commission on Radiological Protection. /3,4/ The concept of weighing the risks and benefits led to the development of standards for the peaceful use of nuclear energy and for the tolerance of patients through medical application of technical sources of radiation. /5/

Our knowledge of the danger of heredity from technical progress can be compared to observing an iceberg. We see the tip of the iceberg. We know the genetic risk of radiation load. The tip of the iceberg only partially indicates the dangers. The scope of genetic hazards due to environmental

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[1] Dedicated to Prof. Otto Hug, M.D., on his 60th birthday.

chemicals is unknown. Although chemically-induced mutations have been known since 1947, /6/ until now we have still not developed any standards for the controlled use of mutagens. One case of the lack of adequate protection regulations is the multiplicity of the decision processes in the evaluation of chemical mutagens. /7/

Mutagenicity of a foreign substance

Chemical mutagens can only be understood by means of a great many methods. /8/ A review of literature by L. Fishbein and colleagues /9/ in 1970 contains 2,719 references for chemical mutagens. These data were laboriously obtained almost exclusively with simple test systems, which have the advantage of multiplying extremely quickly due to their short generation periods. These organisms are further distinguished by a simple genome. With these test systems the molecular principles of mutagenesis can be clarified. /10/

For mutagenicity tests of foreign substances, the question must be answered whether mutation tests with simple test systems make a predication on the mutagen effect of a foreign substance in mammals possible. As a contribution toward the clarification of this question, the same Institute researched the effect of 4 alkylating agents in neurospora, /11/ in cell cultures of Chinese hamsters, /12/ in a host-mediated assay (neurospora-mice) /11/ and mice. /13-17/ Methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), N-methyl-N-nitroso-nitroguanidine (MNNG) and 2-methoxy-6-chlor-9-

/3-(ethyl-2-ethyl chloride) aminopropylamine/acridine dihydrochloride (ICR-170) induce intragenic mutations in neurospora as well as in the cell cultures of Chinese hamsters. In the host-mediated assay system, (neurospora and mice) only MMS and EMS induce mutations. Dominant lethal mutations and specific locus mutations in mice were induced by MMS and EMS. The mutability of cell cultures corresponded to the effect of these agents in neurospora, but not the mutagenicity of mammals. Mutagenicity tests of foreign substances would be much easier if it were possible to identify a network of simple test systems which makes possible greater feasibility of evidence of the mutagen effect of these agents in mammals. Extrapolation is difficult because mammals have a highly developed system for the metabolization and detoxification of foreign substances.

The analyses of chemically-induced chromosome mutations /18,19/ and of the molecular mutation mechanism /10,20/ with simple test systems are important decision aids in the choice of agents which are to be tested in mammal research. /21/ M.S. Legator /22/ emphatically postulated an evaluation of the genetic risks in mammal research. The recommendation of the World Health Organization concurred in this point of view. /23/ The advantages and disadvantages of the various testing methods were reviewed in detail in the Skokloster Report, "Evaluation of Genetic Risks of Environmental Chemicals." /24/

Mutagenicity in Mammals

Chromosome mutations can be determined with cytogenetic methods, which were described in detail in "Chemical Mutagenesis in Mammals and Man." /25/ The cytogenetic methods make possible a direct comparison of mammal research and findings in humans. /26/27/ The relevancy of cytogenetic research for mutation tests is determined with model substances. /28-30/

A less technical effort is needed to achieve dominant lethal mutations in mice or rats which arise as a result of structural or numerical changes in the chromosomes. /31,32/ For research on the induction of dominant lethal mutati ons in mice, a foreign substance is applied to the male animals intraperitoneally, subcutaneously or orally. The test substance can also be put into food or drinking water. After application of the foreign agent, the treated males are mated with the untreated females in 4- or 7-day cycles for 8 weeks. This test arrangement makes the identification of the various germ cell stages possible. If the development of germ cells is not influenced by the treatment of the mice, then the sperm eggs are fertilized and exposed as spermatozoa (1-7 days), spermatids (8-21 days), spermatocytes (22-42 days) or spermatogonia (> 42 days). /33/ The day of mating can be determined by the formation of a vaginal clot. The contents of the uterus of the female are examined 14-17 days after conception. The induction of dominant lethal mutations leads to the death of the

fertilized eggs before or after implantation. The percentage of dominant lethal mutations is determined by the proportion of living embryos per female in the experimental group to the living embryos per female in the control group. /13/

Production of dominant lethal mutations in spermatozoa and spermatids is extremely easy. The method is very sensitive and reliable. The evaluation of the spermatocytes and spermatogonia requires additional research for the determination of the pre-implantable egg losses, when the fertilization frequency in the experimental groups lies below the corresponding control standard. /34/ In this case, the question must be clarified whether the observed reduction of the embryonic litter size is caused the induction of mutations or by the blocking of spermatogenesis. /35,36/

The determination of dominant lethal mutations in the female sex /37/ is hindered by the necessary distinction of genetic and somatic effects. The toxic effect of the test substance can damage the egg cells, block the implantation, or impair the development of the implanted eggs. The systematic comparison of the induction of dominant lethal mutations in female and male mice for MMS, EMS, MNNG and ICR-170, showed that the maximal tolerable dose in both sexes leads to the same final conclusion. /13,17/ An increased amount of testing of dominant lethal mutations in the female sex is only justified in exceptional cases.

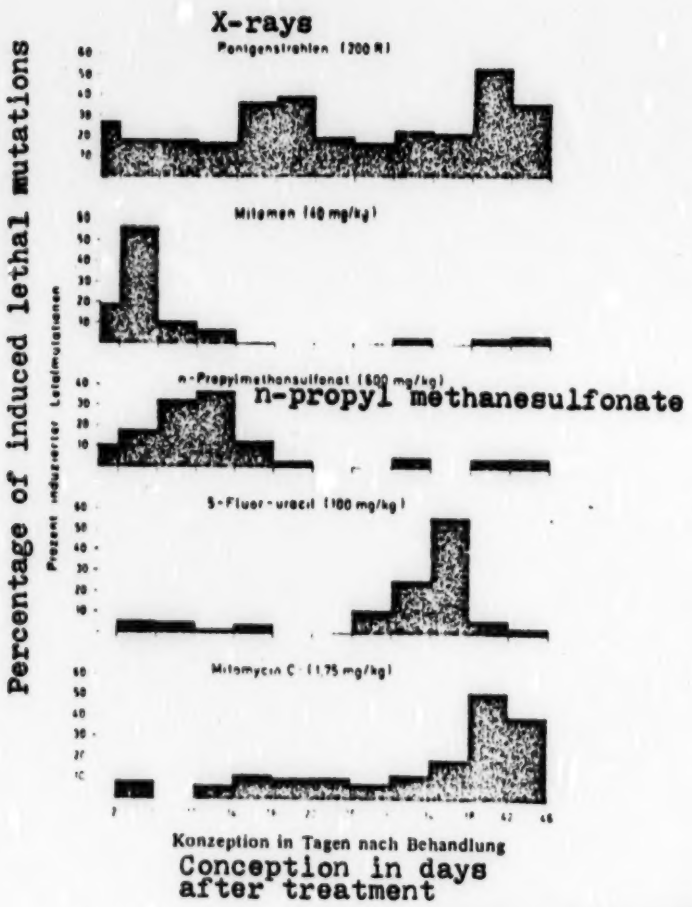
Genetic radiation research showed /38/ that dominant

lethal mutations in all stages of spermatogenesis are induced where the sensitivity of the various germ cell stages is different. (Ill. 1) Chemical mutagens induce, characteristically, dominant lethal mutations only in certain germ cell stages. (Ill. 1) The specific stage effect of the mutagen can be clarified by the biochemical alteration of conditions during spermatogenesis /38,39/ or by a mending process. /40/

Mitomen /41/ induces dominant lethal mutations mainly in spermatozoa. In a test group of 20 males and females with the same dose and mating interval there were deviations in our tests up to 8% within the range of biological variability. (Ill. 1) The manifestation of dominant lethal mutations occurs before or after the implantation.

n-propyl methanesulfonate (PMS) induces dominant lethal mutations exclusively in spermatozoa and spermatids. The spermatids react more acutely to PMS than the spermatozoa. (Ill. 1) Dominant lethal mutations are manifested before and after implantation. /42/ The same specific stage reaction is characteristic for MMS and EMS. /13/

5-fluorouracil (FU) reacts only with the spermatocytes. (Ill. 1). FU inhibits the DNA synthesis by delaying the thymidylate synthetase reaction. /43/ Blocking of the DNS synthesis of the spermatocytes leads to oligospermia through which a lethal effect is simulated. /36/ 5-fluoro-2-deoxyuridine (FUdR) also blocks the development of spermatocytes. /43/



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Ill. 1. Induction of dominant lethal mutations after one-time irradiation or intraperitoneal injection of chemical mutagens in male mice. The varying action mechanisms of the mutagens lead to induction of mutations in different germ cell stages. The criteria for the evaluation of dominant lethal mutations in spermatocytes are elucidated in detail in the test. In contrast to chemical mutagens, ionizing radiation induces mutations in all germ cell stages.

Mitomycin C (MC) blocks, by latticelike polymerization of the complementary DNA strands, the meiotic as well as the mitotic DNA synthesis. In both germ cell stages, dominant lethal mutations /34,38/ as well as oligospermia /35/ were induced by MC. Independent of the blocking of the DNA synthesis, the induction of dominant lethal mutations by MC in spermatids takes place. With higher doses (3.5-7.0 mg/kg) this reaction is substantially more pronounced than in Ill. 1.

The marked specificity of chemical mutagens to react only with certain germ cell stages makes a posteriori conclusions possible on the action mechanism of the test substance. The problems of induction of dominant lethal mutations by isopropyl methanesulfonate (IPMS) in mice was discussed in detail. /42/

To achieve gene mutations, 2 methods were discussed, the host-mediated assay /44/ and the specific locus method on mice, developed by W.L. Russell. /45/ In the host-mediated assay, microorganisms, neurospora-conidia, or other simple test systems were injected into the mammal intraperitoneally, while the test substance was applied subcutaneously, intravenously or orally. This form of application makes it possible for the indicator systems of the potential mutagen to be exposed under the in-vivo conditions of the mammal. After a certain reaction time, the indicator system is obtained from the peritoneal region of the mammal and the mutation production determined. /46/ With the

specific locus methods, 7 different recessive mouse genes were produced in the first offspring. In the mutation experiments, wild colored mice with dominant normal alleles are given the test substance. After treatment, the wild colored mice are mated with an untreated white mouse of the test group, which is homozygous for 7 recessive genes. When, in the germ cells of the treated mouse there are no mutations induced in the test genes, all of the first generation progeny are wild colored. If a dominant test gene mutates, then the mutant is purely hereditable for the corresponding recessive feature and deviates in a characteristic manner from the appearance of the wild colored mouse in the mutation experiment. /15/

In the specific locus experiment of mice, four compounds were researched, chosen according to the following criteria: Mitomycin C and Myleran induce dominant lethal mutations in post-spermatogonial germ cell stages in mice /38,47/ and temporarily inhibit the development of spermatogonia. /48, Tab. 2/ N-methyl-N-nitroso-nitroguanidine (MNNG) and 2-methoxy-6-chlor-9/3-(ethyl-2-ethyl chloride) amino propyl-amino/dihydrochloride (ICR-170) induce no dominant lethal mutations in mice. /13/ Both compounds are strong mutagens in neurospora and induce base pair transitions, respectively, base pair deletions and insertions. /10,11,13/ From these compounds, which are injected intraperitoneally, only MC induces specific locus mutations. (Tab. 1) In spermatogonial

germ cell stages, after application of MC, the mutation rate in comparison to the control standard is significantly increased. (P=0.03) The mutation rate is dependant upon the applied dose. /48/ The mutation rate of spermatogonia after i.p. injection of 4 mg/kg ICR-170 is not significantly increased. (P=.22) After an i.p. injection of 50 mg/kg MNNG or 20 mg/kg Myleran, no specific locus mutations have been observed up to now. Of special interest is the observation that the age of the male, at the appearance of spontaneous mutations in the control animals (Tab. 1) was 460 and 585 days, respectively. An indication of the relation of age frequency to spontaneous mutations was found by G. Schlager and M.M. Dickie. /49/ If these observations confirm a large range of material, then the results of B.M. Cattanach /50/ must be reinterpreted. In humans, an increase in mutation frequency is observed in the ages of fathers with chondrodysplasia. /51,52/

For the induction of dominant lethal mutations, the germ cell stage specific result of the chemical mutagens is characteristic. (Ill. 1) Up to now research with the specific locus method showed that dominant lethal mutations and specific locus mutations were induced into the same germ cell stages. /14-17,48/ For a rational choice of mutagenicity tests, the question must be clarified whether these results can be generalized. In comparing the mutagenic effectiveness of alkylating agents and ionizing

radiation, the germ cell stage specificity of mutation induction must be considered since an undifferentiated approach leads to faulty conclusions. /53/

Table 1

Induction of specific locus mutations in mice by intraperitoneal injection of foreign substances

Compound [1]	Dose (mg/kg)	Post-spermatogonial stages		Spermatogonia	
		No. of progeny	No. of mutations	No. of progeny	No. of mutations
MNNG	50	894	0	7,408	0
Myleran	20	911	0	9,884	0
ICR-170	4	1,092	0	9,275	2
MC	4	277	0	2,759	0
	5.25	10,263	1	20,711	6
	6	2,165	0	2,618	1
	7	1,580	0	1,114	1
Control		32,425			

[1] MNNG (N-methyl-N-nitroso-nitroguanidine)
Myleran (1,4-bis-(methane-sulfonoxy)-butane)
ICR-170 (2-methoxy-6-chloro-9-/3-(ethyl-2-ethyl chloride) amino propylamino/acridine dihydrochloride)
MC (Mitomycin C)

Of principle significance for the evaluation of the risks of chemical mutagens is the question of whether there is a connection between induction of a sterile phase by temporary inhibition of spermatogonia development and induction of specific locus mutations in spermatogonia. Temporary sterility is very easy to demonstrate and can be determined in specific

locus experiments as well as dominant lethal tests. In our chemical genetic tests, isopropyl methanesulfonate (iPMS), Myleran and Mitomycin C induced temporary sterility. (Tab. 2) A positive correlation between temporary sterility and induction of specific locus mutations in spermatogonia is characteristic for MC, 2,4,6-trisethylene imino-1,3,5,-triazine (TEM) and X-rays. /48,50,54/ The correlation with iPMS is questionable. After an i.p. injection of 50 mg/kg iPMS, so far one mutant in 7,178 progeny was observed. /14/ On the basis of results in Myleran tests (Tab. 1 and 2) it could be concluded that there is no causal correlation between induction of a temporary sterile phase and induction of specific locus mutations. According to our present knowledge, however, induction of a temporary sterile phase must be considered a potential risk.

The contrast of the mutagenicity research of MC, MNNG (Tab. 1) and TEM /50/ in specific locus experiments in mice, and the host-mediated assay of salmonella-mice, /55/ shows that the results of both methods are contradictory. (Tab. 3) The predicability of the host-mediated assay could be improved by additional research in the light of further indicator systems. In the host-mediated assay of neurospora and mice, MNNG does not induce mutations. /11/ There is the question of which predication is relevant for mutagenicity tests. The clarification of the contradiction can only ensue through specific locus mutation. The host-mediated assay should

Table 2

Chemically- and radiation-induced temporary sterility of male (101 x C3H)F_1 mice

Mutagen	Dose	Duration of pre-sterile stage (weeks)	Duration of sterile phase (weeks)
Isopropyl methanesulfonate	50 mg/kg	5	3
	200 mg/kg	5	>50
Myleran	20 mg/kg	5	4
	40 mg/kg	4	>50
Mitomycin C	4 mg/kg	4	5
	6 mg/kg	4	6
X-rays	600 R	4	5
	800 R	2	10

complement, but not replace specific locus tests in mice. A mutagenicity test of foreign substances with the specific locus method is absolutely necessary with mutagens, which exclusively induce point mutations in simple test systems. Tests in males could be limited to research on the mutability of spermatogonia, since this germ cell stage is decisive in the risk evaluation of a foreign substance.

The estimate of genetic risk of foreign substances in humans is directed toward the extrapolation of the results of tests on mammals. The problems in transferring the results of radiation genetics in mammals to humans are relatively simple in comparison to the problems relating to the extrapolation of chemical genetic data from mammals. In order to improve

the transferrence of test results, chemical genetic work with small mammals must be supplemented by comparable toxicological research.

Table 3

Comparative mutagenicity testing of foreign substances

	Compound [1]		
	MC	TEM	MNNG
Salmonella	-	-	+
Host-mediated assay (Salmonella mouse)	-	-	+
Specific locus mutations in mice	+	+	-

[1] MC (Mitomycin C)
TEM (2,4,6,-Trisethyleneamino-1,3,5,-triazine)
MNNG (N-methyl-N-nitroso-nitroguanidine)

Genetic Hazards from Foreign Substances

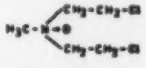
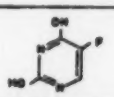
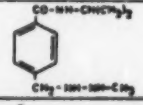
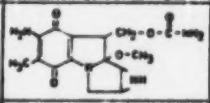
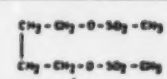
Bryan Bridges called the evaluation of genetic hazards from environmental chemicals "the impossible problem." /7/ This statement is understandable when we consider the number of medications, food additives and industrial chemicals, as well as the production figures. /56,57/

In the evaluation of a foreign substance, which probably produces mutagens in humans, the correlation between benefit and risk is crucial. With mutagens which have no advantage,

the decision is easy. Their use must be prohibited. In mutagens with a benefit, it is desirable to develop structural analogies which possess the positive properties, but have no undesirable toxicological side effects. /21/ The greater the benefit of a compound, the more differentiated the risk analysis must be carried out. The cancer chemotherapeutic agents in Ill. 2 react in dominant lethal research with different germ cell stages. These agents are now being tested in specific locus experiments. When specific locus mutations and dominant lethal mutations are induced into the same germ cell stages, with similar therapeutic efficiency of the medication, there are clear references for the reduction of genetic risk. Especially recommendable would be the cancer chemotherapeutic agents which do not induce any mutations in spermatogonia. An important aid in mutagen decisions, which induce spermatogonia mutations is the clarification of the dose-effect relation in specific locus tests in mice.

The risk analysis requires research on the tolerance of certain individuals or the whole population of the mutagen in question. For research on radiation tolerance, these problems are typically solved. /58/ The estimate of the mutagenic load from medication or food additives is possible. /59/ Research of the load from industrial chemicals is temporarily incomplete.

In evaluating genetic hazards in the environment, there is the danger that the lack of knowledge of a risk will be

Compound	Formula	Spermatozoa (1-7 d)	Spermatids (18-21 d)	Spermatocytes (22-42 d)	Spermatogonia (>42 d)
Verbindung	Formel	Spermatozoen (1-7 Tage)	Spermatiden (8-21 Tage)	Spermatozyten (22-42 Tage)	Spermatogonien (>42 Tage)
Methyl-bis-[2-chlor-aethyl]-amin-N-oxid [Mitomen]		◆	–	–	–
5-Fluor-uracil		–	–	◆	–
N-Isopropyl-α-[2-methyl-hydrazino]-p-toluamid [Natulan]		–	◆	◆	◆,–
Mitomycin C		–	◆	◆	◆
1,4-Bis-[methansulfonyl-oxy]-butan [Myleran]		◆	–	◆	◆

Ill. 2. Sensitivity of the spermatogenesis stages in mice after intraperitoneal injection of cancer chemotherapy agents. A positive reaction in spermatozoa and spermatids marks induction of dominant lethal mutations. The positive reaction in spermatocytes and spermatogonia can be caused by induction of dominant lethal mutations or by blocking of the development of these germ cell stages. Germ cell stages which do not react with cancer chemotherapy agents are marked with a minus sign.

accepted, and that an analysis of the hazard will lead to energetic demands for bans against it. This tendency dominates discussions on the peaceful application of nuclear energy. /60/ In order to avoid misconception in debates on potential harmful substances, mutagenicity tests must be intensively pursued.

Mutagenicity tests require research of the dimension which Alwin Weinberg appropriately described as the concept of "Big Biology." /61/ For the admission of new environmental chemicals, the producer would have to defray the additional costs of mutagenicity tests. Mutagenicity tests of medications, food additives and industrial chemicals on the market must be openly pursued, since these tests were not considered necessary until now and further, research on the interaction of various chemicals with simultaneous application, and the influence of radiation damage from foreign substances. /19/ The degree of synergistic effects in mammals is vastly unknown. The present results /34/ demonstrate the necessity to thoroughly study these relationships. New means are necessary for research in the required dimension. Pursuit of mutagenicity tests must be supplemented by the integration of these methods in the toxicological evaluation of foreign substances. /24,62,63/

Efforts in the identification of carcinogens are probably reduced appreciably by mutagenicity tests. For clarification of the correlation between mutagenicity and

carcinogenicity, neurospora, /64,65/ fungi /66/ and salmonella /67/ were utilized. This interesting research has the same problems which mark the extrapolation possibilities of mutagenicity tests with simple systems. /66/ In dominant lethal research with mice, 5 carcinogens were tested in our laboratories. (Ill. 2) It is interesting to compare these test results with the data of Dietrich Schmähl. /68/ The criterion for the latency period was a 50 percent tumor growth in the rats. The induction period in months was varied for the test substances: Mitomen (16±4), 5-Fluorouracil (10±3), Natulan (14±6), Mitomycin C (18±4) and Myleran (22±4). The control standard was 23±5 months. In order to arrive at a clear statistical predication, the efforts expended in these tests are appreciably greater than in mutation tests with mammals. /41/

Tolerable Load

For mutagens whose benefits are greater than the potential hazards, whose load was ascertained for the population, the tolerable genetic risk must be established. This latter task of mutagenicity tests of foreign substances is particularly difficult. The concept of the recommendable load for radiation-induced mutations in humans was developed by the International Commission on Radiological Protection. The ICRP concept rests on the consideration that there is a balance between spontaneous mutation rates and selection. /1/

In order not to alter this balance radically, the number of induced mutations must be smaller each generation than the number of spontaneous mutations in each generation. /3/ These limits of a tolerable load also apply to the development of standards for the controlled use of chemical mutagens.

Ideally, the controlled use of chemical mutagens presupposes knowledge of all mutagens and the population load of mutagens. This demand is utopian. One possibility of improving the unsatisfactory situation would be the expansion of the sphere of duties of national and international commissions in radiation protection. This form of organization would make possible research on the total load of the population by ionizing radiation and environmental chemicals and could correspondingly adapt the standards of the improved knowledge. The integrated approach would be preferable to the arbitrary division of the load in radiation and chemical genetic components. The methods of mutagenicity tests of foreign substances are indeed imperfect, but sufficient to decisively reduce genetic risks to future generations. This view leads to the demand to provide the technical conditions now which make possible the determination of chemical genetic hazards from environmental chemicals. Standards for the controlled use of chemical mutagens can be developed by balancing the risks and benefits. Clarification of radiation genetic risks demonstrates how research on chemical genetic hazards can ensue. The number of potential harmful

substances to be tested requires many times the effort than was required for radiation genetics.

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